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Review - Environmental Sciences

The Zebrafish as an Alternative Animal Model for Ecotoxicological Research and Testing

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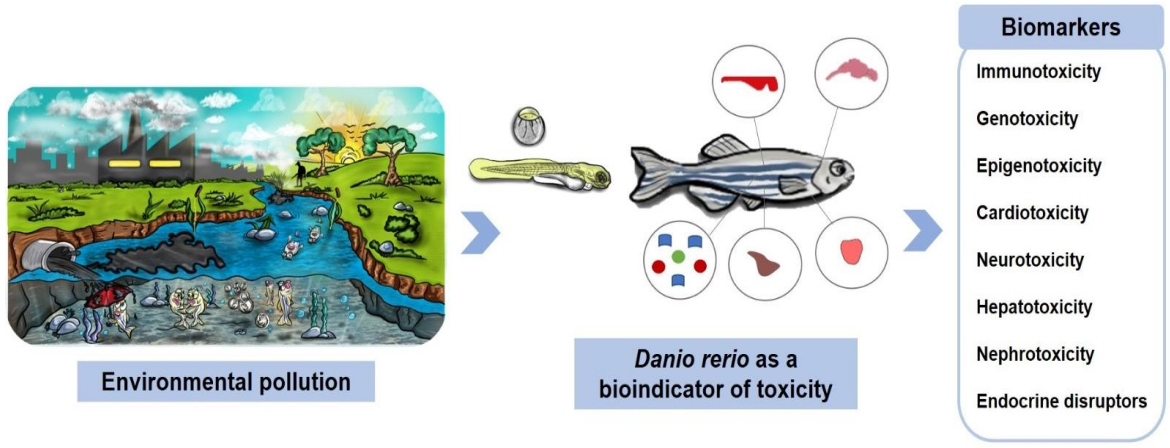
HIGHLIGHTS

- Biomarkers of toxicity in zebrafish.
- Toxicological screening with the embryonic, larval, and adult stages of zebrafish.
- Scientific advancements in the assessment of the effects of environmental contaminants.

Abstract: Anthropogenic interventions have had a compromising effect on environmental health, intensifying the degradation of ecosystems, and the quantity of chemical pollutants released into nature. Therefore, research areas within the scope of environmental assessments and monitoring such as ecotoxicology have contributed to the determination of the toxic potential of contaminants. A small cyprinid known as the zebrafish (*Danio rerio*), the use of which has exponentially grown, is an alternative vertebrate model for scientific research, mainly in the assessment of environmental risks. The species exhibits several advantages for breeding in a laboratory, in addition to presenting multi-biomarkers of environmental toxicity. Thus, this review aims to present the main characteristics and advantages of working with this species, as well as show studies related to ecotoxicology involving biomarkers of toxicity in zebrafish. The results show a progressive trend towards employing the species in environmental risk analyses, it is an increasingly recommended species in the assessment of the toxicity level of a range of chemical pollutants. The development of future technologies must contribute to scientific advancement, rendering the potential application of this model organism an even more widespread one, which will certainly help in bridging knowledge gaps in various areas of study.

Keywords: Ecotoxicology; toxicity biomarkers; environment pollution; *Danio rerio*; model organism.

GRAPHICAL ABSTRACT



INTRODUCTION

In recent decades, an imbalance in anthropic activities has led to a degradation of the environment, this being one of the biggest socio-environmental problems around the world [1]. These practices greatly contribute to the release of new chemicals into the environment, which are often able to interact with Earth's physical and biological components, even interfering with its natural environmental cycles [2]. The main sources of environmental contamination include the disposal of solid waste, the release of untreated effluents, and the indiscriminate use of agrochemicals in agriculture [3]. Moreover, tannery processes, pharmaceuticals, fossil fuels, and mining industries also represent sectors that contribute concomitantly to the instability of ecosystems [4].

The translational science of ecotoxicology came to the fore with the intention of optimizing environmental assessments and monitoring. The tests allow researchers to elucidate the response of organisms to exposure to different test substances [5]. In addition, ecotoxicological analyses are based on the protection of ecosystems and the prevention of environmental contamination [6]. One of the most sensitive bioindicators of environmental change is the fish species [7]. For this reason, they are models widely applied in toxicology studies and are considered essential animals for research, especially in the field of ecotoxicology [8]. Besides the economic and ecological importance, fish are excellent model organisms because they have several biomarkers of contamination, that superimpose on mammalian models [9].

The zebrafish embryos (<96 hours old) are considered an alternative model as it conforms to the 3Rs of animal experimentation [10]. Since zebrafish embryos easily absorb molecules inserted into their water, the first studies with the species were already based on the teratogenic effects in embryos previously exposed to chemical substances [11,12]. Since then, zebrafish have been used as a tool in toxicological analysis [12]. Among the main features that make *D. rerio* an excellent model for such assessments is their short life cycle, rapid attainment of reproductive maturity, short and external embryonic development, transparent embryos, high egg permeability, low cost of laboratory maintenance, ease of reproduction, and the high quantity of eggs produced by the female [13]. Furthermore, the species shares 70% of orthologous genes with humans and has body plan similarities, making it additionally a promising model for molecular studies [14].

The mode of action of test substances in *D. rerio* can be understood through analyses with embryos, larvae, or adult fish. The evaluation parameter follows different approaches since the species is a strong model for addressing emerging questions in toxicology, such as the influence of xenobiotics on the onset of health and disease [15]. As a result, zebrafish have become popular within several lines of biological research and present biomarkers related to different organisms' physiological systems and chemical modes of action [16].

Some aspects pertaining to the zebrafish reproduction and embryo-larval tests will be discussed below. Subsequently, some of the most relevant research areas that use zebrafish as an animal model will be discussed. Thus, this review was carried out to present the advantages and use of zebrafish as an animal model in scientific research. We focused, with great emphasis, on the different biomarkers of environmental assessment of the species, and advances of its applicability in experimental ecotoxicology. (Figure 1).

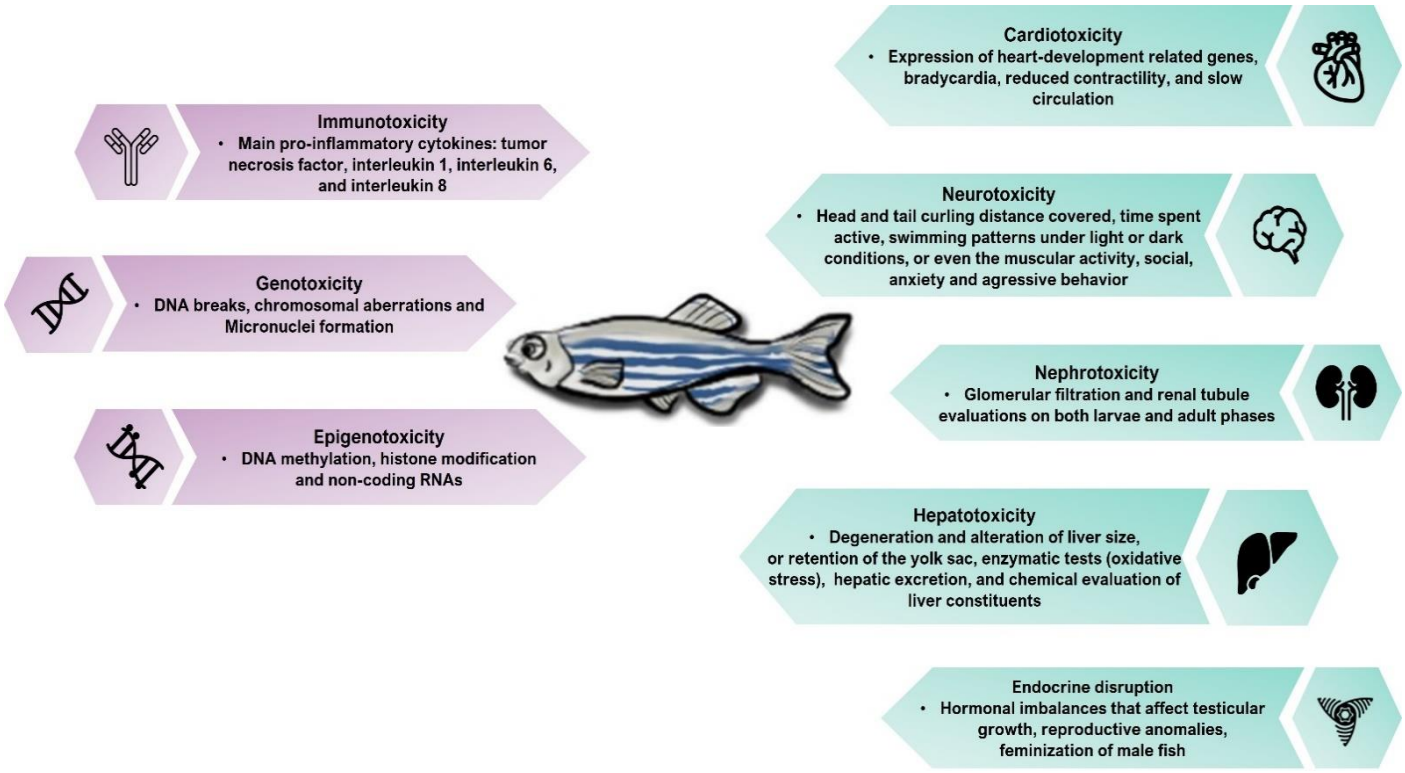


Figure 1. Schematic representation of the main toxicity biomarkers using zebrafish as an animal model.

In recent decades, the zebrafish embryotoxicity test has gained a good scientific reputation, especially in toxicological assessments. It is considered a low-cost and easy-handling test when compared to those of other vertebrate models [17]. The zebrafish embryo toxicity assay has shown a high correlation of results with mammals, including humans [18]. Furthermore, it is an experiment that makes it possible to reduce the use of higher vertebrates, and presents methodological refinement, reducing injuries and the mistreatment of laboratory animals [19].

The direct visualization of the embryo phases *in vivo* allows the evaluation of the effects of different compounds in the first 96h of life [20]. Exogenous substances easily interfere with the natural development of embryos during ontogenesis [21]; and because they are whole organisms, the permeability of the chorion by small molecules can occur in the first stages of life, a process unlikely to be found in cell culture systems [22]. The main phases of zebrafish development are illustrated in Figure 2.

According to OECD (test 236), zebrafish embryos are exposed to the test substance at the blastula stage (3hpf). The toxic level of the test substance is characterized by parameters such as egg coagulation, hatching rate, pericardial edema, lack of pigmentation, body length, heartbeat, and malformations of the head, tail, and yolk sac [23]. With the aid of a stereomicroscope, the parameters are analyzed every 24h for a total period of 96h (OECD, 2013). At the conclusion of the test, an effective concentration (EC_{50}), or even a lethal concentration of the test substance (LC_{50}), may be estimated, thus evaluating the level of teratogenic effects in zebrafish.

Recent research shows the ecotoxicological effects of a range of contaminants in the early life stages of zebrafish development [24–26]. Low concentrations of polyethylene microplastic had a negative effect on the embryonic development of *D. rerio* [16]. The authors reported a significant difference in egg hatching rates and larval survival up until the end of exposure (96 hpf) [16]. Pharmaceuticals are a threat to the environment and are often found in even higher concentrations than pesticides in river sediment samples [27]. Researchers reported the severe teratogenic effect of drugs in the early development of zebrafish, demonstrating acute toxicity with greater lethality in the first 24 hpf [27].

Malformations in zebrafish larvae exposed to the insecticide Fipronil induced decreased larvae length, heart defects, and diminishment of egg hatchability occurred [28]. Ecotoxicological effects on *D. rerio* embryos are also reported as a result of exposure to herbicides [29]. The authors have elucidated the effects of Orbencarb, an herbicide widely used in the agricultural sector. The results showed that the substance negatively affected the organogenesis of previously exposed individuals [29]. Furthermore, Orbencarb compromised embryo viability and induced morphological abnormalities in the brain, yolk sac, and spinal cord of zebrafish larvae [18].

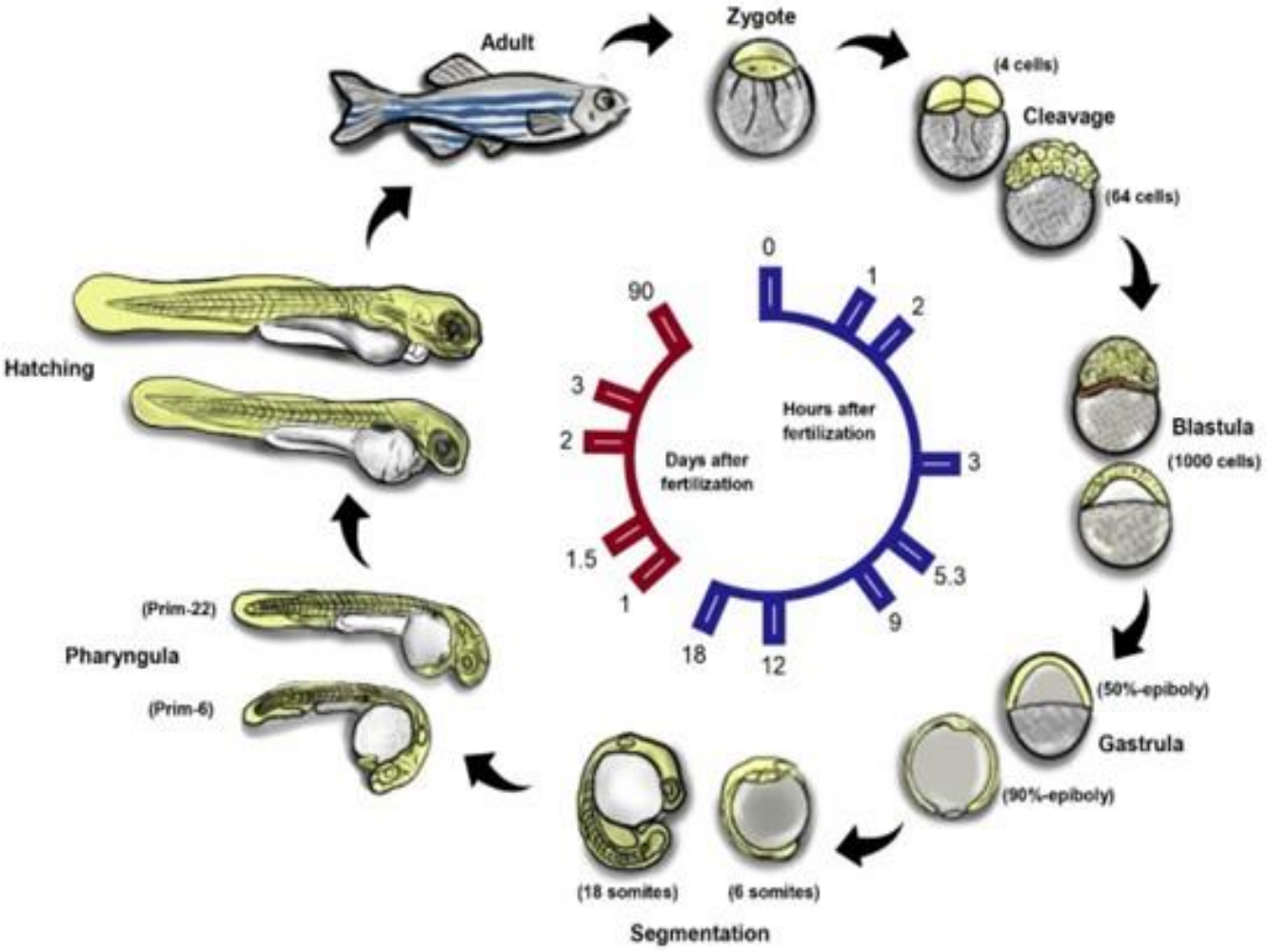


Figure 1. Life cycle and main stages of zebrafish development

TOXICOLOGICAL STUDIES

Immunotoxicity

The zebrafish has been reported as a robust animal model for *in vivo* immunology studies [30,31]. The transparency of the fish in the larval period enables the visualization of fluorescence-labeled molecules. Thus, it is possible, for example, to monitor the migration of cells to the site of infection [32]. From an immunological point of view, zebrafish possess cells, receptors, and cytokine expression that are also found in humans. There are 19 putative genes for Toll-like receptors (TLR) in zebrafish [33].

A disturbance of the immune system due to exposure to environmental contaminants can lead to serious alterations in the environmental balance pathogen-host: once the system is disturbed, innate cellular immune responses may fail to defend against infections [34].

The main pro-inflammatory cytokines are currently in use as immunotoxicology markers against various environmental contaminants [31]. Exposure of zebrafish embryos to concentrations lower than 3 mg/L of cypermethrin (pesticide) can induce the transcription of genes related to cellular apoptosis and immunotoxicity [35]. Studies used zebrafish larvae to determine immunotoxicity against exposure to graphene oxide, using the expression of tumor necrosis factor (TNF-alpha), interleukin 1 (IL-1), and interleukin 6 (IL-6) as biomarkers. The results of this study showed a significant increase in the expression of these cytokines after 14 days of exposure to graphene oxide at different concentrations [30].

Contaminants such as atrazine, a pesticide widely used in various agricultural crops, can cause altered expressions of cytokines such as TNF-alpha, interleukin 1 (IL-1), interleukin 6 (IL-6) and interleukin 8 (IL-8) [31]. In a study in which zebrafish embryos were exposed to the herbicide Butchlor, it was possible to observe that the mRNA level of genes related to innate immunity in embryos was significantly induced by Butchlor.

The expression of cytokine genes such as IL-lß, CXCL-C1c, CC-chem, and IL-8 in early development was induced dependently on the Butchlor concentration. The cytokine IL-1ß acts on the recruitment of neutrophils and macrophages at the site of the lesion. On the other hand, the cytokines IL-8, CXCL-C1c, and CC-chem are inflammatory mediators [36].

Genotoxicity

The most widely used approaches in genotoxicity testing involve the identification of endpoints, such as DNA breaks, chromosomal aberrations, and micronucleus frequency [37]. In view of this, for the study of xenobiotic genotoxicity, the zebrafish model has also proved to be an excellent one and has been used in tests to evaluate the most diverse compounds through assays that identify such endpoints.

The Comet Assay is a test that allows the identification of DNA breaks, one of the endpoints of genotoxicity, to take place. It is a test characterized as sensitive and efficient in detecting the genotoxic potential of various substances and can be performed on a variety of organisms, including zebrafish [38]. Tests performed with environmental samples [39], pesticides [56] and metals [41] confirm the use of the zebrafish, in various forms (embryos or cells isolated from specific tissues), as a sensitive model for the detection of damage to genetic material [42].

Another test capable of identifying the genotoxicity of substances is the Micronucleus Test (MN). The MN test can identify both the clastogenic and aneugenic effects of compounds, in addition to being highly sensitive and predictive, easy to perform, and applicable to different cell types [43]. Through the MN test, it is also possible to use zebrafish as a model for evaluating genotoxicity in various contexts, such as in water quality assessment [44], in water pollution, as a form of environmental monitoring [45], in the development of new drugs [46], and in the promotion of conscious and safe use of products [47].

However, it is more common to use a combination of both tests, which is the best way to identify the mechanism of action of a genotoxic substance, as well as the response of the body's repair system. Studies performing the Comet assay and MN Test in zebrafish liver cells found a positive response for the former; that is, the compound-induced DNA breaks. However, the authors state that the lesions were repaired, as it was not possible to detect the presence of MN in the cells [48]. In contrast, some researchers were able to correlate the two genotoxicity endpoints positively when studying the effect of the Spent Pot Liner (SPL), a residue generated during aluminum production, on zebrafish erythrocytes, since the induction of genetic material fragmentation and the increase in the frequency of MN suggested a clastogenic effect on the compound [49].

Epigenotoxicity

Several studies using zebrafish have already demonstrated its effectiveness in the investigation of morphological and behavioral changes. However research that characterizes the mechanisms behind these changes and their transmission through generations is scarce [50]. Among these mechanisms are those that affect gene expression without altering the nucleotide sequence of DNA, which are identified through epigenetic changes, some of which are inherited by different generations [51]. The main ways by which gene expression can be epigenetically affected are DNA methylation, histone modification, and non-coding RNAs [52]. Recently, studies that have linked epigenetic changes with exposure to toxic substances and environmental pollutants have identified several substances capable of altering these mechanisms [53].

DNA methylation occurs when a methyl radical ($-CH_3$) is added, most commonly onto the carbon 5 of a cytosine, transforming it into a 5-methylcytosine. This phenomenon may result in the silencing of the gene corresponding to the methylated region [54]. The use of zebrafish is advantageous for understanding DNA methylation because its protein machinery involved in the process is similar to that of mammals [55]. For example, zebrafish have genes orthologous to those of mammals for the so-called DNA methyltransferases (DNMTs), enzymes responsible for adding the methyl group to DNA, especially in CpG dinucleotides [56].

Bisphenol A, a chemical utilized in the production of epoxy resins and polycarbonate plastics, induced alterations in behavioral parameters, such as swimming. Such alterations are attributed to changes in the methylation patterns of nervous system-related genes [57]. In another study, the investigation of the epigenetic effects of the insecticide Fipronil showed that the deregulation of genes involving *D. rerio* development was related to their hypermethylation induced by the compound [58], corroborating the idea that this epigenetic mechanism is responsible for gene silencing [59].

Additionally, it was also possible to observe the transmission of epigenetic alterations throughout the generations in zebrafish. It was demonstrated when exposing *D. rerio* embryos to different concentrations of methylmercury (MeHg), that generations not exposed to the contaminant showed both phenotypic effects (hyperactivity and visual impairment) and epigenetic changes in male gametes, which occurred through the increase of differentially methylated regions in genes associated with actin and cytoskeleton [60].

There are also records of the use of zebrafish in studies involving other epigenetic mechanisms, such as histone modification. Such modifications occur by the addition of radicals and are called acetylation, methylation, and ubiquitination. Acetylation is associated with gene activation since it acts by making the chromatin structure more accessible to transcription factors. Methylation can act either by activating or inhibiting gene expression, depending on the number of radicals added and the position in which they are inserted into the histone tail, while ubiquitination is characterized by inhibiting gene expression [61].

Studies using Bisphenol A showed that in both males and females, it was possible to observe changes in this epigenetic mechanism and how it had a direct influence on zebrafish reproduction. In females, the deregulation of reproduction-related genes, responsible for the maturation of oocytes, was a consequence of changes in the chromatin structure due to the modification of histones involved in transcription activation and gene silencing [62]. In males, the transmission of histone hyperacetylation patterns from the paternal epigenome to the progeny was observed, impairing the development of the embryo [63].

It is also possible to find in the literature studies that use *D. rerio* as a model for studying a third epigenetic mechanism: non-coding RNAs (ncRNAs). These RNAs, transcribed molecules that are not translated into proteins, play an important role in gene regulation through interaction with DNMTs and histones [64]. These studies focus on some ncRNAs present in zebrafish that are conserved among vertebrates, especially in humans. A study focused on the effects of Atrazine on the expression of miRNA-126, which is related to the process of angiogenesis during Zebrafish development. The author observed that the expression of this RNA was sensitive to compound exposure and might result in changes in the processes related to the formation of blood vessels and related structures [52]. Table 1 summarizes the results presented in the toxicity studies (immunotoxicity, genotoxicity, and epigenotoxicity).

Table 1. Effects of pollutant exposure on zebrafish.

Substance	Concentration	Effect	Reference
		Immunotoxicity	
Cypermethrin (pesticide)	<3 mg/L	Induce the transcription of genes related to cellular apoptosis and immunotoxicity	[35]
Graphene oxide	1, 5, 10 and 50 mg/L	Significant increase in the expression of cytokines TNF-alpha, IL-1 and IL-6	[30]
Atrazine (pesticide) and metabolites	30, 100 and 300 μgL^{-1}	Altered expressions of cytokines TNF-alpha, IL-1; IL-6 and IL-8	[31]
Butchlor (herbicide)	0, 4, 6, 8 and 12 µM	Expression of cytokine genes IL-lß, CXCL-C1c, CC-chem, and IL-8) in early development	[36]
		Genotoxicity	
Sewage effluents	samples from 6 sites	DNA damage measured by Comet Assay	[39]
Cd and carbon nanotubes	Cd alone and in co-exposure with oxidized multiwalled carbon nanotubes	DNA damage measured by Comet Assay	[41]
4-nitroquinoline-1-oxide (model genotoxicant)	0, 0.1, 0.3, 1.1, and 2.9 µg/l	Induced an unscheduled DNA synthesis (UDS test); DNA fragmentation; reduced cell viability (Alkaline filter elution technique); DNA fragmentation in gills and hepatocytes (Comet Assay); increase of micronuclei (Micronucleus test)	[42]
Aquatic mixture	22 Water samples from the Danube River	Significant micronucleus induction	[44]
Naturally occurring metals in groundwater	Fe (0.8 and 1.3 mg/L); Mn (0.2 and 0.4 mg/L). Groundwater collected from deep tube wells with Fe (0.8/Mn 0.2 mg/L) and Fe (1.3/Mn 0.4 mg/L)	Increase in the frequency of micronucleus in all samples	[45]
Ulexite	40 mg/L	Genotoxic damage observed by the Micronucleus assay and Oxidative DNA demage	[46]
Formalin	T1: 0.45 mg L^{-1}; T2: 4.57 mg L^{-1} T3: 22.86 mg L^{-1}	Increase in the average number of micronuclei in peripheral blood erythrocytes	[47]

Cont. Table 1

Anticancer drugs	Cyclophosphamide (CP) and ifosfamide (IF) individually and in a mixture with 5-fluorouracil and cisplatin	CP and IF induced DNA break and genomic instability at high, concentrations (environmentally irrelevant); Mixture induced an increase in DNA break at low concentrations (for environmental contamination)	[48]
Spent Pot Liner (SPL)	0.32; 0.64; 0.95 g L^{-1}	DNA fragmentation Frequency of micronuclei and damaged nucleoids increased with increasing SPL concentration	[49]
		Epigenotoxicity	
Bisphenol A	10 µM	Changes in methylation patterns of nervous system-related genes	[57]
Fipronil (inseticide)	100; 200; 400 800 µg/L	Hypermethylation of genes related to *D. rerio* development	[58]
Methylmercury (MeHg)	1; 3; 10; 30; 100 nM	Generations not exposed to the contaminant showed both phenotypic effects and epigenetic changes in male gametes (through the increase of differentially methylated regions)	[60]
Bisphenol A	5; 10; 20 µg/L	Deregulation of reproduction-related genes due to histone modification	[62]
Bisphenol A	100; 2000 mg/L	Transmission of histone hyperacetylation patterns from the paternal epigenome to the progeny, impairing embryo development	[63]
Atrazine	0.3; 3; 30 ppb	Affected the expression of miRNA-126, related to the angiogenesis process	[52]

SPECIFIC ORGAN TOXICITY

Cardiotoxicity

Zebrafish and mammals share similar functional characteristics of the heart, including the directing of blood flow, a high-pressure system governed by endocardial musculature, a regulated heart rhythm, and heartbeats associated with pacemaker activity. The potential morphology and basic contractile dynamics of this cardiac system, analogous to those of humans, allow the zebrafish to be a well-established model in experiments that focus on channelopathies and cardiomyopathies. The zebrafish heart has also revealed age-related changes in cardiac structure and function, such as myocyte hypertrophy, ventricular fibrosis, and valvular lesions. Furthermore, the pharmacological responses of the species to cardiotoxin exposure are similar to responses obtained in humans [65].

The embryos have a heart composed of two chambers that remains visible during the first week of development. Transgenic strains of zebrafish, which express fluorescent proteins exclusively in the myocardium, have been developed to assist in monitoring the high heart rate in the species [66]. In addition to transparency, which enables the direct observation of cardiac function, the zebrafish larval stage offers benefits in the study of the cardiovascular system regarding electrophysiological maturation and heartbeat rhythm, characteristics that reach stability from 96 hpf [67]. The *ex-situ* development also contributes to experiments involving transplantation, a technique considered laborious but very promising for the investigation of cellular autonomy. This technique has recently been expanded to include transparent adult zebrafish [68].

The evaluation of gene expression in zebrafish can contribute to an extrapolation of the effects of tested compounds that induce cardiotoxicity, due to the conservation of molecular mechanisms between this species and human beings. Pyrimethanil fungicide-induced changes in the transcription of apoptosis-related genes (p53, Bax, Bcl2, Casp 9, and Casp6l1) and heart development-related genes (Tbx2b, Gata4, Myh6, Vmhc, Nppa, Bmp2b, Bpm 4 and Bpm 10) in zebrafish [69]. Other examples of genetic markers selected in cardiotoxicity studies in this species include SORBS2, RXRA, DNAJB6, and ANO5 [70].

Other authors have also investigated the cardiotoxic action of different groups of chemical compounds in zebrafish. This includes effects such as bradycardia, reduced contractility, and slow circulation provoked by medications (mitoxantrone, terfenadine, clomipramine, and thioridazine [71]; damage to the cardiovascular system caused by the pyrethroid insecticide deltamethrin [72]; and cardiac toxicity brought about by exposure to preservatives such as triclosan and polycyclic aromatic hydrocarbons (PAHs) [73].

Neurotoxicity

The global occurrence of neurological diseases has progressively increased due to several factors, including the rise in environmental contamination caused by neurotoxic substances present in the environment. Therefore, the assessment of neurotoxicity induced by chemical agents is an important challenge due to the morphological and physiological complexity of the central and peripheral nervous system [74]. The zebrafish has become a well-established and widely used model in the investigation of neurological and behavioral damage from different exposures to xenobiotics [75].

The neurotoxicological effects observed in zebrafish embryos can be evaluated in relation to their spontaneous movement, initiated at around 19-26 hpf, and characterized by head and tail curling [76]. During the larval stage, their behavior can be evaluated through the distance covered, time spent active, swimming patterns under light or dark conditions, or even the muscular activity produced in response to a loud sound, known as acoustic startle [77]. The evaluation of these parameters together can lead to the characterization of complex behaviors such as hypolocomotion, hyperlocomotion, motor incoordination, and sickness behavior, among others [78].

In adulthood, *D. rerio* individuals present socially interactive behavior in groups, having the ability to distinguish their conspecifics by the general appearance, including color, shape, and stripe pattern [79]. Social behavior tests in adult zebrafish were developed based on individuals' habituation, interaction, and reaction to social stimuli. Throughout the test, the fish are allowed to swim freely, and their social preferences may be assessed by measuring the time spent by fish in the zones of social stimulation [80]. Short-term exposure to oxybenzone in adult zebrafish induced individuals to spend less time interacting with the shoal due to a loss of social motivation, instead of the motor deficits caused by the exposure to the studied pollutant [81].

In addition, other authors have reported anxiety-like larval behavior through thymotaxis, shown by the individuals having a preferred location at the edge of the aquarium [82]. The escape response to threatening stimuli was another behavior analyzed during this period [83]. Likewise, the neurotoxicological effects caused by xenobiotics are commonly investigated in adult zebrafish individuals; for example by inducing anxiety and testing aggressive behavior [84].

The neurodevelopment of zebrafish shows similarities with the human species [85], in addition to its having neuroanatomical structures and organization homologous to those among mammals in the developing brain [86]. The species also has telencephalic neuroanatomical regions, an optic tectum, and thalamus, hypothalamus, cerebellum, olfactory bulb, and a spinal cord [87]. In this context, the presence of glial cells, astrocytes, oligodendrocytes, Purkinje cells, and myelin and neuronal circuits similar to those observed in mammals was also identified [88].

The incorporation of chemical substances in the brain is restricted by the blood-brain barrier (BBB), present in mammals, responsible for maintaining homeostasis in the brain [89]. Zebrafish have a BBB similar to that of higher vertebrates (3 dpf), and as its embryonic and larval development occurs ex-utero, access to the central nervous system is facilitated. Therefore, an animal model with a BBB resembling that found in mammals is of great interest for studying neurotoxic diseases in humans [90].

Hepatotoxicity

The liver is an essential organ that performs numerous activities, including being the site for synthesizing blood proteins, such as clotting factors, and detoxifying xenobiotics, toxins, and drugs [91]. Due to its role in toxin metabolism and its sensitivity to environmental pollutants, this organ has been highlighted in toxicological studies related to contamination by organic and inorganic chemical agents [92].

Toxic substances that cause injury or functional disorders to the liver are called hepatotoxins and are generally related to environmental contamination and pharmaceutical uses, such as metals, persistent organic compounds, hydrocarbons, pesticides, detergents, and drugs [93]. These compounds can be easily absorbed by vertebrates and invertebrates in various ways, such as in breathing or feeding, and in addition to bioaccumulation in organs and tissues, they can generate metabolic and enzymatic variations [94]. Among the liver changes caused in the presence of toxic substances, zonal necrosis, hepatitis, cholestasis, steatosis, granuloma and even neoplasia stand out [95].

The zebrafish is a promising model for the evaluation of hepatotoxicity mechanisms and has been widely used in studies of environmental contaminants and the pharmaceutical industry as well as in research related to behavioral biology [96], pharmacology [97], and toxicology [98]. More sensitive liver toxicity studies

generally use *in vivo* tests, as they allow the study of sample toxicity within the physiology of an entire organism [99].

The zebrafish liver has every cell type that the mammalian liver possesses, except for Kupffer cells, which are hepatic immune cells. However, it performs essential functions such as glycogen storage, metabolism of xenobiotics, and secretion of bile and serum proteins. In this way, it performs the same functions as the human liver [100]. It is fully functional at only 5 dpf, allowing the *in vitro* study of fish larvae in hepatotoxicity assays [101].

In a work using zebrafish larvae, the hepatotoxicity of six hepatotoxic drugs in mammals and two non-hepatotoxic compounds was evaluated, considering specific hepatotoxic activities, such as degeneration and alteration of liver size, or retention of the yolk sac. The work showed that all hepatotoxic drugs in mammals induced hepatic degeneration, reduced liver size, and delayed yolk sac absorption in zebrafish, while non-hepatotoxic drugs did not generate adverse effects, presenting a similar rate of results when compared to those in mammals, highlighting the zebrafish as a successful model in the overall prediction for hepatotoxic drugs [91].

There are various methods for evaluating hepatic toxicity in zebrafish, such as the visual assessment of macroscopic and microscopic morphological changes, in addition to enzymatic tests, hepatic excretion, and chemical evaluation of liver constituents [102]. Antioxidant enzymes can be increased or decreased under chemical stress, being essential for the conversion of ROS into harmless metabolites [103]. The increase in reactive oxygen species (ROS) and the excessive accumulation of antioxidant enzymes are associated with oxidative stress in aquatic organisms, which negatively affects macromolecules such as lipids, proteins, and nucleic acids. Furthermore, it influences the imbalance between oxidation and the antioxidant system, resulting in excessive oxidation [104].

Some authors demonstrated a significant increase in the antioxidant enzymes superoxide dismutase, catalase, and peroxidase in the liver of male and female fish exposed to the fungicide tebuconazole [104]. The pesticide thiophanate-methyl also showed hepatotoxicity in *D. rerio* and induced disturbances in liver metabolism by activating caspase-3 and oxidative stress. The authors mention the gradual accumulation of ROS and the increased activity of catalase and superoxide dismutase in the groups exposed to the contaminant [105].

A study evaluating the potential toxic effect of microplastics on zebrafish found that after seven days of exposure, the compound accumulated in their livers. Histopathological analysis showed that the microplastics caused inflammation and accumulation of lipids in the animals' livers, in addition to a significant increase in the activity of catalase and superoxide dismutase enzymes, indicating oxidative stress [106]. Long-term exposure to the broad-spectrum fungicide, Flutolanil was also studied using zebrafish as a study model. In this work, fish were exposed to different concentrations of the chemical (0, 0.25, 50, and 1000 µg/L). The results obtained on hepatotoxicity showed that catalase activity fell in all groups treated with the compound and some pathological changes in the liver occurred, including hepatic vacuolization [108].

Nephrotoxicity

The kidney is an important excretory and regulatory organ; it becomes therefore particularly vulnerable to xenobiotic activity. Once it undergoes changes, it is unable to perform its main function, namely, removing toxic molecules from circulation. It has a very complex anatomic composition and a great variety of cell populations. In mammals, for example, the kidney has more than 20 cell population types, including epithelial and mesenchymal cells [98]. Due to the many important functions performed by this organ, monitoring biomarkers that indicate kidney failure and histopathological aspects have become popular among toxicological bioassays [109].

Since the zebrafish is a freshwater species, the main function of its kidney is water excretion and osmoregulation. Even so, the similarities between zebrafish and mammal kidneys make the former an excellent experimental model for renal research, being mostly used for investigations of glomerular filtration and renal tubule evaluations on both larvae and adult phases [110]. A study showed that microcystin-LR (MCLR) induced nephrotoxicity in adult zebrafish. After a 60-day exposure to different concentrations of MCLR (0, 1, 5, and 25 µg/L), histopathological lesions such as renal tubules filled with eosinophilic casts, abnormal renal tubules, intertubular space decreased, and blood infiltration in renal cells was observed [111]. When investigating the nephrotoxicity of acetaminophen (n-acetyl-para-aminophenol, paracetamol) in zebrafish embryos at different developmental stages, researchers showed that between the control group and acetaminophen-exposure group (2.25 mM), no morphological changes were observed in zebrafish

kidneys. However, after exposure to higher dosages (22.5 and 45 mM), the kidneys showed signs of malformation, such as a curved and cystic pronephric tube, pronephric duct, and cystic and atrophic glomerulus. The authors concluded that malformed kidney phenotypes are dose-dependent [112].

Aristolochic acid (AA), a compound used in phytotherapy drugs, induced damage in zebrafish kidneys. It was observed that at 15 days post-fertilization exposed for eight days to 0.5 μM AA, zebrafish kidneys showed clear signs of acute injury, which led to a loss of function in the glomerular filtration barrier [84]. A study that approached the nephroprotective effects of resveratrol and ursolic acid, demonstrated that those substances can attenuate the AA-induced malformations, concluding that zebrafish is an efficient model for assessing nephroprotective compounds [84]. Other studies on zebrafish kidneys address their regenerative characteristics, including the ability to replace epithelial populations after acute injury and to develop new renal functional units called nephrons [113].

Endocrine system disruptions

In recent times, a significant increase has been noted in deviations from normal physiological development and in the incidence of rare diseases [114], which may be triggered by endocrine system disruption, leading to negative effects, as the individual is exposed to substances known as endocrine disruptors (EDs) [115]. According to the U.S. Environmental Protection Agency, endocrine disruptors are exogenous chemicals that can interfere with the homeostasis of natural hormone synthesis, secretion, transport, binding, and elimination of the organism [116].

These persistent organic pollutants are heterogeneous and may be classified as natural or synthetic. Among the best-known synthetic EDs are polychlorinated biphenyls, polybrominated biphenyls, bisphenols, benzophenone phenols, polychlorinated and polychlorinated aromatic hydrocarbons, industrial EDs, plastics, pesticides, fungicides, cyclic synthetics, and chemical and household products [117].

The excretion of crude endocrine-disrupting compounds and their metabolites in water and sewage treatment plants may be the main route of contamination of water resources [118]. Studies show that even after all the stages of wastewater treatment, ED agents of the estrogen class and others can be detected in the final product, due to the inefficiency of their removal during treatment [119].

The contact of fish with the abovementioned ED hormones can lead to hormonal imbalances that affect testicular growth, reproductive anomalies, feminization of male fish, and consequent reduction of population [120]. Additionally, such substances have a bioaccumulative potential and can reach the fish in two different ways: direct participation through inhalation exposure or via the diet through trophic transfer [121].

Zebrafish have been one of the most utilized aquatic bioindicators to perform toxicity tests that evaluate the effect of endocrine-disrupting compounds, due to their useful characteristics as a biological model [122]. The polycyclic aromatic hydrocarbon Benzo(a)pyrene induces mortality and the premature hatching of zebrafish. Polychlorinated hydrocarbons also generate harmful effects on the species, among which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) stands out [123]. According to one study, malformations and increased mortality were identified in the subsequent offspring of zebrafish fish during an embryogenesis experiment. Deformities were also observed in the cranial and axial skeleton during the adult phase, and failures in reproduction, with a clear decrease in egg production and feminization of the population, where fish with distinctly feminine aspects carried testes instead of ovaries [124].

Bisphenol A (BPA) is a potential ED, used commercially for the manufacture of epoxy resin and polycarbonate plastics; therefore, ecotoxicological tests are extremely important for assessing their effects on organisms. The exposure of *D. rerio* to three different concentrations of BPA for 15 days demonstrated both a reduction in global DNA methylation and in the expression of the enzyme DNA methyltransferase 1 (DNMT1), besides interrupting stages of the fish reproductive processes [125]. As well as interfering in these processes, BPA induced a significant increase in the production of reactive oxygen species and lipid peroxidation, resulting in disturbances in membrane fluidity, in addition to the loss of its mitochondrial integrity and functionality [126].

Table 2 summarizes the results presented in the toxicity studies (cardiotoxicity, neurotoxicity, hepatotoxicity, nephrotoxicity, and endocrine system disruptions).

Table 2 - The effects of pollutants on specific organs of zebrafish.

Substance	Concentration	Effect	Reference
		Cardiotoxicity	
Pyrimethanil (fungicide)	2, 4, and 6 mg/L	Changes in the transcription of apoptosis-related genes (p53, Bax, Bcl2, Casp 9, and Casp6l1) and heart development-related genes (Tbx2b, Gata4, Myh6, Vmhc, Nppa, Bmp2b, Bpm 4 and Bpm 10)	[69]
Deltamethrin	50; 25; 10; 1; 0.1 mg/L	Damage to the cardiovascular system	[72]
Triclosan and Polycyclic Aromatic Hydrocarbons (PAHs)	0.4; 40; 400 µg TCS/L	Pericardial edema, altered heart structure and regurgitation (backflow across the heart valve)	[73]
		Neurotoxicity	
Triclosan	62.5; 125; 250 µg/L	Neurotoxicity for oligodendrocyte precursor cells (OPCs)	[77]
Oxybenzone	10; 100; 1000 µg L^{-1}	Reduced locomotion, decreased anxiety-like behavior, less time near/interacting with the shoal, fewer interactions with the mirror image, and decreased exploration of the novel arm in the T-maze test	[81]
Buspirone; Chlordiazepoxide; Caffeine	25; 50; 100 mg/L 0,1; 1; 10 mg/L 25; 50; 100 mg/L	Anxiety-like larval behavior	[82]
		Hepatotoxicity	
Acetaminophen, Aspirin, Tetracycline HCl, Sodium valproate, Cyclophosphamide, Erythromycin and two non-hepatotoxic compounds: Sucrose and Biotin	Four concentrations: 1/10 Maximum non-lethal concentration (MNLC); 1/3 MNLC; MNLC and LC_{10} of each drug or compound	Hepatotoxic drugs induced liver degeneration, reduced liver size and delayed yolk sac absorption in larval zebrafish. Non-hepatotoxic compounds did not have observable adverse effect on zebrafish liver	[91]
Tebuconazole	1/10 MNLC, 1/3 MNLC, MNLC, LC_{10})	Alterations in liver histoarchitecture. Increases in reactive oxygen species levels.	[104]
Thiophanate-methyl (pesticide)	6.25; 12.5 and 25 mg/L	Disturbed liver metabolism, activated caspase-3 and induced oxidative stress	[105]
Polystyrene microplastics	5µm and 70nm	Inflammation and accumulation of lipids in the animals' livers. Increase in the activity of oxidative stress enzymes	[106]
Flutolanil (fungicide)	0.25; 50; 1000 µg/L	Activity decreases of oxidative stress enzymes, such as catalase and pathological changes including hepatic vacuolization	[108]
		Nephrotoxicity	
Microcystin-LR	1; 5; 25 µg/L	Histopathological lesions (renal tubules filled with eosinophilic casts, abnormal renal tubules, intertubular space decrease, and blood infiltration)	[111]
Acetaminophen (n-acetyl-para aminophenol, paracetamol)	22.5 and 45 mM	Kidney malformation (curved and cystic pronephric tube, pronephric duct, and cystic and atrophic glomerulus). The effects were dose-dependent	[112]
Aristolochic acid (AA)	0.5 µM	Acute injury (led to a loss of function in the glomerular filtration barrier)	[84]
		Endocrine system disruptions	
Water samples from a wastewater dominated stream	Complex mixture of Endocrine Active Compounds (EAC)	Hormonal imbalances affecting testicular growth, reproduction, inducing feminization of male fish, and consequent reduction of population	[120]
Sulfentrazone	0.0100; 0.0400; 0.400 mg/L	Influenced steroid biosynthesis	[122]
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	50 pg/ml	Feminization of the population (appearance of fish with clearly female bodies, yet carrying testes in place of ovaries)	[124]
Bisphenol A	0.01; 0.1; 1 mg/L	Increased egg production, and reduced rate of fertilization. Altered the transcription of genes involved in reproductive function and epigenetic processes.	[125]
Bisphenol A	2, 10 and 100 µg/L	Lower sperm motility and alterations on velocity parameters of spermatozoa	[126]

OMICS TECHNOLOGIES

During the last decade, significant advancements have occurred in omics technologies, which have been extensively employed in various research domains. Omics analysis is divided into distinct fields, including genomics, transcriptomics, proteomics, and metabolomics (Figure 3). These technologies possess the capacity to generate high-throughput datasets, offering comprehensive information on genes, transcripts, proteins, and metabolites, respectively [127].

From a toxicological perspective, omics serve as valuable tools for identifying biomolecular alterations induced by exposure to chemical agents [128]. In comparison to previous approaches, omics techniques present notable advantages, notably the capability to comprehend molecular-level changes by leveraging a wealth of pertinent information. Moreover, these techniques hold the potential to enhance chemical safety assessment and decrease the reliance on animal testing in the field of regulatory toxicology. Consequently, they enable a more comprehensive, precise, and expedited investigation in the assessment of environmental toxicity [129].

The utilization of zebrafish as an animal model has demonstrated promising potential in integrating omics technologies, particularly in the realm of toxicological research. Genomics, for instance, can unveil alterations in genes and gene regulatory processes following exposure to toxic compounds [130]. Adverse effects were observed in adult zebrafish exposed to a polystyrene nanoplastic at its lowest concentrations (0.01 and 0.1 mg/L) [131]. The authors assessed the long-term effects and concluded that there were deficiencies in genetic regulation, indicating inflammation of the skin and gills, dysbiosis of the intestinal microbiota, as well as a diminished reproductive capacity.

Techniques associated with transcriptomics center on the comprehensive and systematic investigation of RNA transcripts within a cell, tissue, or organism. During the translation process, mRNA functions as a transcript, and the quantification of the transcriptome can be accomplished through sequential analysis of the nucleotides present in the expressed mRNA. Microarray and next-generation sequencing (NGS) are technological approaches that have the capability to provide comprehensive information about the transcriptome [130]. For instance, we can cite a study where toxicity was assessed by sequencing the transcriptome (RNA-seq) of zebrafish in response to exposure to carboxymethylcellulose-stabilized iron sulfide nanoparticles [132]. The exposure induced notable alterations in the expression of genes associated with immune and inflammatory responses, detoxification processes, oxidative stress, and DNA damage/repair.

Proteomics is a valuable tool for investigating both natural diseases and chemical toxicity, as it enables the identification of protein biomarkers and the elucidation of molecular events that occur following exposure to xenobiotics. Therefore, the primary objective of proteomics is to identify the structure, function, and modifications of proteins [133]. Moreover, the compilation of proteomic data using the zebrafish animal model can serve as a valuable resource for future studies focused on protein networks and biological evolution. Zebrafish complete genome sequencing enables the discovery and characterization of proteins through proteomics, leveraging integration with existing databases [134]. Proteomic analysis unveiled that benzyl benzoate had a significant impact on proteins involved in the biosynthesis of organonitrogen compounds, translation, lipid transport, stress responses, and cytoskeletal activity [135].

Metabolomics is the systematic study of endogenous metabolites and biochemical processes within cells, tissues, or organisms. This technique aims to identify and characterize the end products of toxic reactions. In this manner, it enables the comprehension of changes in metabolic pathways, biochemical interactions, and molecular modifications, along with the identification of pertinent biomarkers for toxicity [136]. The method for analyzing metabolites can be categorized as a target or non-target analysis, wherein they are extracted and identified using analytical techniques based on mass spectrometry [130]. Recently, a study was conducted to assess the impact of exposure to indoxacarb at a real environmental concentration on the liver of adult zebrafish [137]. Metabolomics results demonstrated that the levels of amino acid-related metabolites were impacted following exposure. Additionally, the downregulation of glutathione metabolism resulted in a decrease in the liver's detoxification capacity, indicating a relatively high level of toxicity.

Single omics-based approaches have limitations providing only a narrow view of biomolecular variations, thereby impeding a comprehensive understanding of toxicity mechanisms. In this regard, the application of multi-omics techniques enables a more comprehensive understanding of toxicity mechanisms [138] (Figure 3).

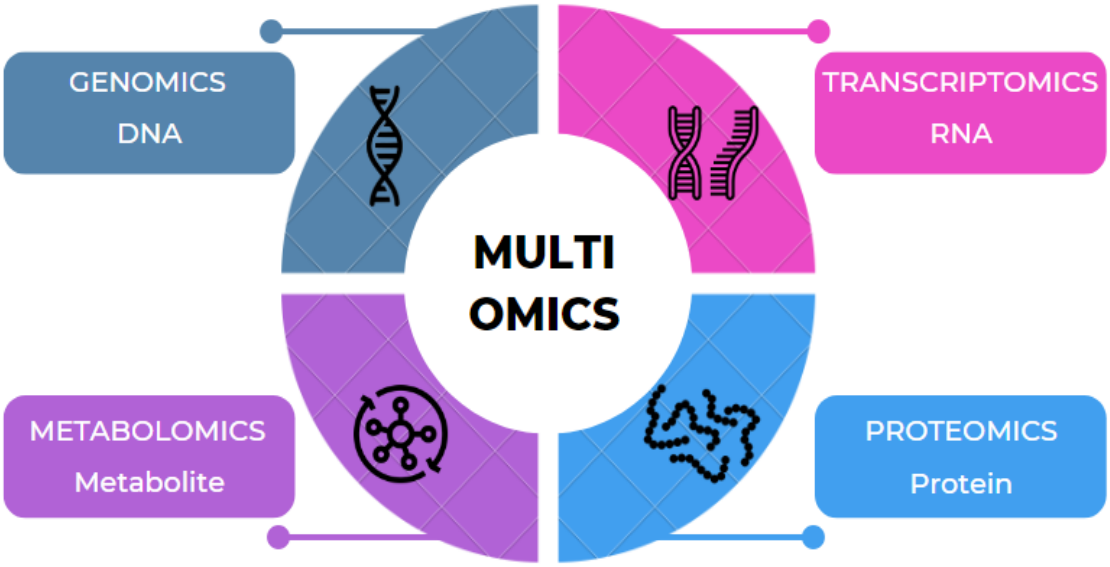


Figure 2. Schematic representation of the applicable omics fields in zebrafish toxicological aspects.

RECOMMENDATIONS AND PERSPECTIVES

The use of zebrafish as a model organism for assessing environmental toxicity, utilizing different biomarkers, has been demonstrated to be a valuable and promising approach. Based on the literature review carried out and, on the biomarkers, used in our study, we present here recommendations and perspectives for future uses of zebrafish.

It is essential to recognize that environmental toxicity manifests at several levels of biological organization, spanning from the molecular to the population level. Therefore, the adoption of multi-scale approaches, integrating studies across molecular, cellular, tissue, and whole-organism levels, is recommended. This comprehensive approach will facilitate a more robust assessment and extrapolation of the effects of contaminants on populations and communities within their natural environment [139].

While our primary focus was on acute exposures due to the heightened sensitivity of zebrafish embryos and larvae to environmental factors, which enables rapid screening for toxic agents, we also incorporated studies involving chronic exposures in adult fish. It is very important to add to the well-established studies with this bioindicator, new technologies that can provide complementary information to the existing ones on the impacts of pollution and, thus, outline a more realistic approach to the consequences of these impacts for aquatic life [140].

To obtain a comprehensive understanding of the effects of environmental pollutants on zebrafish, it is crucial to integrate data from various biomarkers. Adopting a holistic approach that encompasses multi-omics aspects, immune response, gene expression, enzymatic activity, and organ function will enable a more extensive elucidation of both toxic and adaptive effects. This integration of diverse biomarkers will yield valuable insights into the underlying mechanisms driving the impacts of environmental toxicity in zebrafish [141].

Furthermore, to guarantee the comparability and reproducibility of studies, it is imperative to standardize experimental protocols and analysis methods employed in the evaluation of environmental toxicity. Collaborative efforts within the scientific community are essential to establish standardized guidelines and protocols, facilitating the comparison of studies and interpretation of results [142].

The future holds great promise for zebrafish in the field of animal experimentation, and there is a belief that their utilization will expand in the years to come. Emerging research avenues offer the potential for significant scientific advancements with this species, particularly in the assessment of the detrimental impacts of environmental contaminants. For instance, notable progress can be anticipated in the enhancement of omics techniques, as well as the integration of mathematical and computational models in toxicological analyses. These developments will contribute to a more comprehensive understanding of the effects of environmental pollutants on zebrafish and pave the way for innovative research approaches [143].

The zebrafish, particularly its embryos, and larvae, is expected to become increasingly integral in high throughput screening (HTS) of small molecules and drug libraries during the discovery phase of potential new therapeutics. Furthermore, it is essential to establish a 'safe-by-design' approach utilizing zebrafish, which involves modifying the toxic properties of substances to synthesize safe alternatives. Consequently,

by harnessing contemporary and emerging technologies, we can anticipate significant strides in bridging existing knowledge gaps through advancements in research utilizing *D. rerio* as an animal model. This innovative approach holds immense potential for accelerating the development of safe and effective therapeutics [144].

These recommendations and perspectives emphasize critical areas that warrant future investigation in utilizing zebrafish as an animal model for environmental toxicity assessments. By adhering to these guidelines, we can make significant progress towards gaining a more profound comprehension of the risks associated with environmental pollutant exposure. Ultimately, this knowledge will contribute to the safeguarding and preservation of aquatic ecosystems and human health.

CONCLUSIONS

The global acceptance of *D. rerio* as a modern experimental animal model is increasing gradually. As has been seen, the use of zebrafish has recently been elucidating fundamental biological processes in an interdisciplinary manner, covering several areas of study, particularly toxicology and biomedical research, throughout both their adult and embryo-larval stages.

In this literature review, we highlighted the progress of the scientific applicability of the species in the field of environmental toxicology. The studies discussed here are related to its response to exposure to different xenobiotics, involving experiments with the embryonic, larval, and adult stages of zebrafish. This demonstrates the efficiency of this animal model as a tool in ecotoxicological tests, due to the increasingly sophisticated methodologies being developed to elucidate the cause and effect of environmental contaminants.

The present review provides an insight into the current status of ecotoxicological knowledge obtained through zebrafish bioassays. The data presented also show the need for a comprehensive future ecotoxicological assessment with this model. This information will assist in inferring the potential effects of xenobiotics and predicting their potential risks to the aquatic ecosystem.

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